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An HSV*tk*-mediated local and distant antitumor bystander effect in tumors of head and neck origin in athymic mice

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The "bystander effect," produced by ganciclovir-mediated killing of cells transduced with a herpes simplex virus thymidine kinase (HSV*tk*) gene, defines the cooperative killing of non-HSV*tk*-transduced cells. *In vitro*, a major contributor to this phenomenon is metabolic cooperation involving transfer of cytotoxic small molecules between cells via gap junctions. In this study, the bystander effect was assessed *in vivo* using cells of oral squamous cell carcinoma origin. Mixtures of HSV*tk*$^+$ and HSV*tk*$^-$ tumor cells were implanted subcutaneously in the left flank of nude mice, and naive HSV*tk*$^-$ cells were implanted subcutaneously in the right flank. When tumors attained a size of 0.5 to 1 cm, the animals were treated with ganciclovir on a daily basis. The tumors comprised of mixed cells in the left flank resolved, consistent with a predicted bystander effect. The naive tumors in the right flank either resolved or became cytostatic showing little further growth compared to controls. Similar results were obtained when naive tumors were grown in both flanks and the tumor in the left flank received intratumoral injection of HSVtk retroviral producer cells or PA317 (HSV*tk*$^+$) packaging cells, but not parental NIH 3T3 cells. Concomitant treatment with dexamethasone impaired the antitumor effect on the contralateral side. When these experiments were performed in SCID-Beige mice, there was a reduced antitumor effect on the ipsilateral flank and no antitumor response in the contralateral flank. Together with histology of regressing tumors, which showed an infiltration of lymphoid cells, these results are suggestive of an immune-related antitumor response that could account for the distant bystander effect.

Key words: Head and neck tumors; gene therapy; ganciclovir; herpes thymidine kinase; therapy.

The efficiency of retroviral transduction of tumors for gene therapy purposes remains low, resulting in genetic modification of only a small fraction of the tumor cells.[1] Thus, any strategy that does not require all or most of the cells to be transduced represents a significant advantage. One such approach involves transduction of tumor cells with a herpes simplex virus thymidine kinase (HSV*tk*) gene by direct intratumoral injection of producer cells that produce infective, replication defective virus particles that encode the viral thymidine kinase. The transduced tumor cells are rendered sensitive to ganciclovir as are other adjacent nontransduced cells.[2,3] This observation has formed the basis for several cancer gene therapy trials. The transfer of ganciclovir sensitivity from transduced to nontransduced cells has become known as a "bystander effect." The cellular mechanisms underlying the bystander effect *in vitro* alternatively have been ascribed to phagocytosis of apoptotic vesicles[4] and to metabolic cooperation involving transfer of small cytotoxic molecules between cells via gap junctions.[5–8] The mechanisms involved in the bystander effect *in vivo* are more complicated and less well understood. For the *in vitro* bystander effect, we have favored metabolic cooperation as the major participating factor, and have described the transfer of labeled phosphorylated derivatives of ganciclovir from HSV*tk*$^+$ tumor cells to HSV*tk*$^-$ cells by autoradiography.[5] In this report, we have asked whether or not this bystander effect is manifest *in vivo* by subcutaneous injection of mixtures of HSV*tk*$^+$ and HSV*tk*$^-$ into nude mice followed by ganciclovir administration after the tumors had formed. The tumors derived from the mixed cell population resolved or exhibited a reduction in size consistent with metabolic cooperation as a mediator of the bystander effect. However, control naive tumors on the contralateral side of the same mice exhibited reduced tumor growth or reduction in size, or even complete regression in several mice. This observation is consistent with alternative mechanisms such as involvement of the immune system.

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MATERIALS AND METHODS

Cell culture and in vitro *HSVtk transduction*

The human UM SCC29 cells are of human oral squamous cell carcinoma origin and were kindly provided by Dr. Thomas

Carey (University of Michigan). The cells were maintained in modified Eagle's medium (MEM) containing 10% fetal bovine serum (FBS) and 1% nonessential amino acids (Life Technologies Inc., Gaithersburg, Md). The G1TkSvNa7 producer cells were provided by GTI, Inc. (Gaithersburg, Md), and the PA317 packaging cells were a gift from Dr. Dusty Miller (Fred Hutchinson Cancer Center, Seattle, Wash).[9] The parental NIH 3T3 cells were obtained from the ATCC. These three cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and 10% FBS.

The UM SCC29 cells were infected with retrovirions produced by G1TkSvNa7 cells that rendered the target cells HSV*tk*$^+$- and ganciclovir-sensitive, as well as resistant to G418. The infection of UM SCC29 cells was carried out according to a standard procedure.[10] Infected cells were selected in medium containing 1 mg/mL G418, and further tested for ganciclovir sensitivity in medium containing 20 mM ganciclovir (kindly provided by Syntex, Palo Alto, Calif).

For co-culture experiments, cells were plated in equal numbers at a density of 1.4×10^4 cells/cm^2 in 10-cm culture plates in MEM with 1% nonessential amino acids plus 10% FBS. Ganciclovir was added to a concentration of 20 μM. All experiments were carried out in triplicate, and control plates were treated with PBS, the solvent used for ganciclovir. After 5 additional days, cells were stained with trypan blue, and viable cells were counted.

In vivo *tumorigenesis*

Eleven-week-old (22–25 g) outbred Balb/c nude mice (Hsd: Athymic Nude-*nu*) were purchased from Harlan Sprague-Dawley (Indianapolis, Ind) and maintained in Microisolator cages in the College of Medicine animal laboratory facilities. CB17 SCID-Beige mice were obtained from Charles River. All animal experiments were performed in accordance with animal protocols approved by the University of Cincinnati Institutional Animal Care Committee. A total of 5×10^6 UM SCC29 cells were injected into each flank of a series of nude mice, and tumor growth was monitored. By 2 weeks, the tumors had grown to approximately 0.5 to 1 cm in size. At this time 2×10^7 G1TkSvNa7, PA317, or NIH 3T3 cells were injected intratumorally in the left flank. Ganciclovir treatment was initiated 48 hours later by intraperitoneal injection of 50 mg/kg weight twice daily. The tumor size was measured with a caliper on every third day. The tumor weight was estimated by calculating $0.5 \times L \times W^2$ (L = the longer dimension; W = the shorter dimension). The tumor weight at 2 weeks was established and used as a baseline for further tumor growth or regression.

Histology

Tumors were excised from the mice, fixed in Omnifix (An-Con Genetics, Inc., Melville, NY) for 24 hours, embedded in OCT compound, and sectioned with an American Optical 820 microtome. The 5-μm sections were stained with eosin and hematoxylin and examined by light microscopy.

RESULTS

Mixed tumors comprised of HSVtk$^+$ and HSVtk$^-$ cells and naive tumors comprised of HSVtk$^-$ cells in opposite flanks of the same mouse respond to ganciclovir

Before asking whether or not the bystander effect can contribute to tumor regression *in vivo*, it was necessary

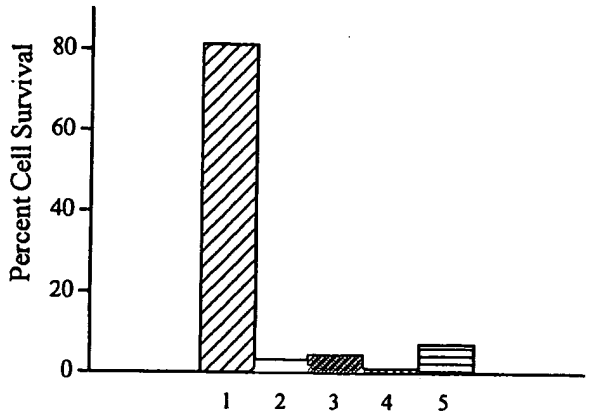

Figure 1. Bystander killing of naive tumor cells *in vitro*. UM SCC29 cells were co-cultured with either UM SCC29*tk* or PA317 cells at a 1:1 ratio in medium containing 20 mM ganciclovir. Each experiment was carried out in triplicate. After 5 days of co-culture, the cells were trypsinized, stained with trypan blue, and counted. The number of surviving cells for each co-culture was normalized to that of the control counterpart co-culture which was treated with PBS. **1:** UM SCC29; **2:** UM SCC29*tk*; **3:** UM SCC29 + UM SCC29*tk*; **4:** PA 317; **5:** UM SCC29 + PA317.

to establish that the UM SCC29 cells were capable of manifesting an *in vitro* bystander effect. HSV*tk* gene modification did not alter UM SCC29 growth properties (unpublished data). Growth of naive UM SCC29 cells was affected slightly by treatment with 20 mM ganciclovir, whereas, administration of ganciclovir to UM SCC29 tk$^+$ cells was cytocidal (Fig 1). Co-culture of UM SCC29 cells with UM SCC29 tk$^+$ cells, at a density where the majority of cells are in contact with one another, resulted in killing of both cell populations (Fig 1), indicative of metabolic cooperation.

To determine whether or not the bystander effect would manifest *in vivo*, HSV*tk*$^+$ cells were mixed with HSV*tk*$^-$ cells at a 1:3 ratio and injected into the left flank of a series of nude mice. As a control, naive UM SCC29 cells were implanted into the right flank. By 12–14 days, the tumors were between 0.5 and 1 cm, at which time a regimen of daily ganciclovir administration was initiated. The tumors derived from the mixed-cell population regressed, and in several cases resolved completely, consistent with a bystander effect mediated by metabolic cooperation (Fig 2A). The tumors in the right flank exhibited reduced growth ($P < .01$), and in some cases also resolved, an observation that cannot be explained by the local effects of metabolic cooperation, but requires alternative explanation.

Intratumoral injection of HSVtk$^+$ packaging cells or producer cells affects tumor growth at local and distal sites following ganciclovir treatment

In an experiment of similar design, naive UM SCC29 cells were implanted subcutaneously in both flanks of nude mice. After 14 days, when the tumors had reached a size of 0.5 to 1 cm, the tumor in the left flank was injected with 2×10^7 producer cells that produce

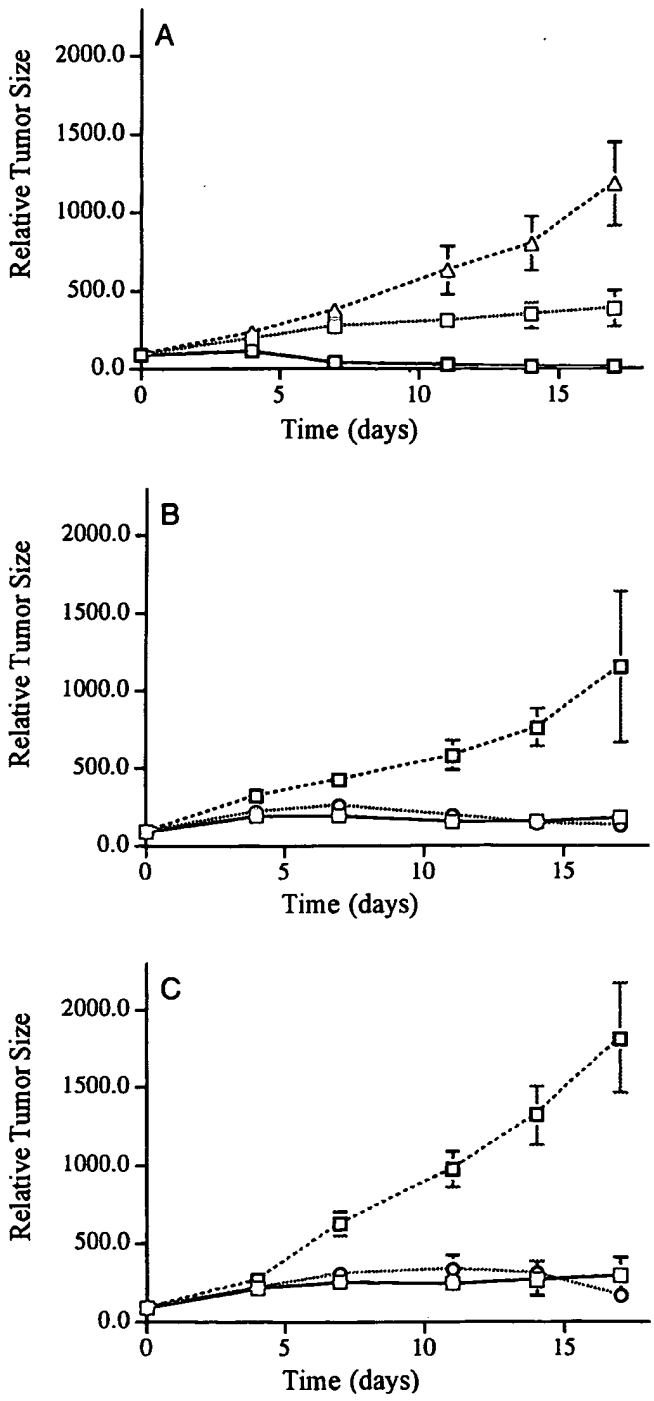

Figure 2. Ganciclovir inhibition of tumor growth in mice with tumors containing HSV*tk*$^+$ cells or following intratumoral injection of HSV*tk* producer cells or PA317 packaging cells. **A:** A 1:3 mixture of UM SCC29*tk*:UM SCC29 cells was implanted subcutaneously into the left flank (ipsilateral; open squares) of 21 nude mice. The same number of UM SCC29 cells (5×10^6) was implanted into the right flank (contralateral; closed squares) of these same mice. As a control, 12 mice were injected with UM SCC29 cells only (closed triangles). Tumors were allowed to grow to between 0.5 and 1.0 cm at which time they were measured and set as a standard for normalization. The tumor sizes were set at 100% and the day of first measurement was designated as day 0. On day 2, ganciclovir administration was initiated and continued for a 2-week period, during which time tumor sizes were periodically measured and recorded. **B** and **C:** Eight nude mice were injected bilaterally with 5×10^6 UM SCC29 cells, and tumors were allowed to form to a size of 0.5 to 1.0 cm. At that time, the tumors on the left flank of 16 mice were injected with 2×10^7 G1TkSvNa7 producer cells (open circles), tumors on the left flank of 16 mice were injected with PA317 packaging cells (closed squares) or of left flank tumors injected with NIH 3T3 cells (open squares). Tumors on the contralateral side remained uninjected. Two days after injection of cells into the ipsilateral tumors, twice daily ganciclovir administration was initiated. **B:** Tumor responses on the ipsilateral flank; **C:** Tumor responses on the contralateral flank.

HSV*tk*-containing retrovirus, and 48 hours later the mice were initiated on a regimen of daily ganciclovir administration. The ipsilateral tumors regressed as in the previous experiment, and the contralateral tumors again exhibited growth inhibition and some regression (Fig 2, B and C). In 7 out of 24 mice, the contralateral tumors resolved.

To determine whether or not virus production was necessary to induce an antitumor response, 2×10^7 PA317 packaging cells were injected into UM SCC29-derived tumors in the left flank while tumors in the right flank were left untreated. The PA317 cells, which serve as retroviral packaging cells, are derived from thymidine kinase-deficient murine fibroblasts.[9] When they were constructed, they were transfected with HSV*tk*, which was used as a selectable marker.[9] However, even though these cells do not produce HSV*tk* retroviruses, they were effective in eliciting an antitumor response following ganciclovir treatment (Fig 2, B and C). As before, the tumors in both ipsilateral and contralateral flanks regressed significantly and sometimes resolved completely ($P < .005$).

The observed antitumor response was neither due to intratumoral injection of murine fibroblasts nor to the administration of ganciclovir. This is evident from data in Figure 2. Naive tumors derived from UM SCC29 cells were injected with parental NIH 3T3 cells from which the PA317 cells were derived. When these mice were subjected to treatment with the same ganciclovir regimen, the tumors showed no evidence of retarded growth but increased in size as rapidly as untreated controls.

To gain insight into how intratumoral implantation of PA317 cells followed by ganciclovir administration can lead to killing of tumor cells, PA317 cells were co-cultured *in vitro* with UM SCC29 cells. The PA317 cells are sensitive to ganciclovir, whereas the UM SCC29 cells are resistant. However, when the two cell lines were co-cultured at a density that allowed cell-cell contact followed by ganciclovir administration, both cell populations were killed (Fig 1). The UM SCC29 cells were killed with an efficiency comparable to that observed when UM SCC29 cells were co-cultured with UM SCC29tk$^+$ cells (Fig 1). These results suggest that human cells of oral squamous cell carcinoma origin can undergo metabolic cooperation with murine PA 317 cells.

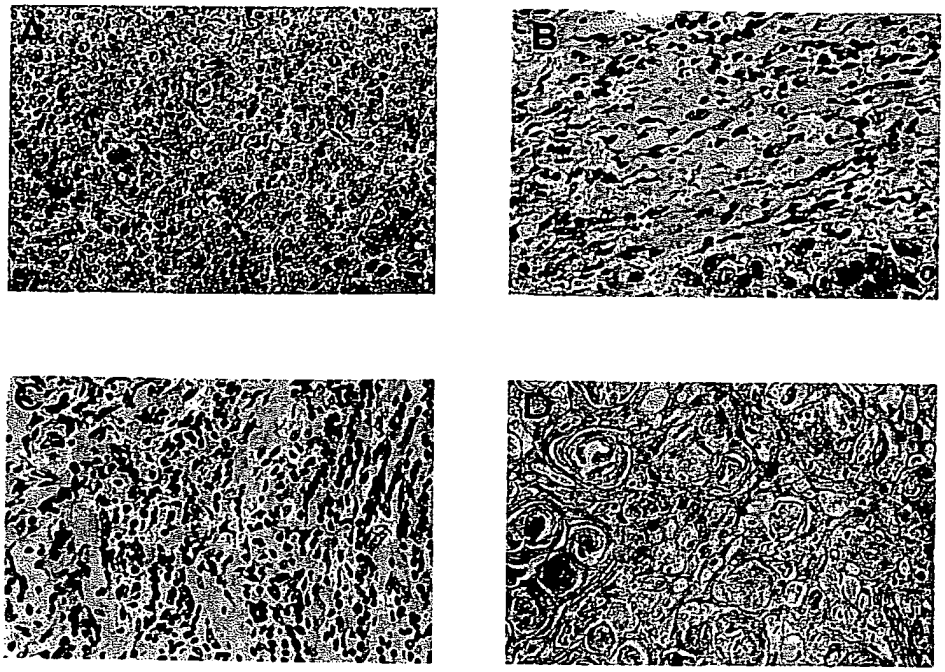

Figure 3. Microphotographs of untreated tumor and tumors in regression or after resolution. **A:** Untreated tumor; **B:** mononuclear cells surrounding tumor tissue (at bottom right); **C:** mononuclear cells at site of resolved tumor; **D:** keratinized pearls at site of resolved tumor infiltrated with mononuclear cells.

Evidence for involvement of an immune response

The response of tumors at sites distant from the treated tumor cannot be explained by the type of metabolic cooperation observed *in vitro*. To better understand the biological mechanism involved, regressing tumors were excised and examined histologically. In all cases assessed, regressing tumors were infiltrated with or surrounded by lymphocytic cells (Fig 3). This cellular infiltration was present regardless of whether the isolated tumor was from the ipsilateral or contralateral side. This observation implies that an immune response was likely an integral participant in the local (ipsilateral side) and distant (contralateral side) bystander effect *in vivo*. To examine the proposition that the observed antitumor effect involves an immunological component, a separate cohort of nude mice bearing bilateral tumors derived from HSV*tk*$^-$ UM SCC29 cells received an intratumoral injection of PA317 cells in the left flank followed by treatment with ganciclovir plus dexamethasone, a suppressor of immune response. Figure 4 shows that, although there was no significant impact on tumor regression was on the ipsilateral side, there was more vigorous tumor growth on the contralateral side in comparison with that without dexamethasone treatment ($P < .05$).

Since nude mice may manifest a residual cell-mediated immune response that could account for the observed antitumor activity, an experiment with a 1:3 mixture of HSV*tk*$^+$ and HSV*tk*$^-$ cells, similar to that described in Figure 2, was carried out in SCID-Beige mice. Tumors grew faster in SCID-Beige mice than in nude mice, and required only 8 days to reach 5-mm diameter compared to 12–14 days in nude mice. As shown in Figure 5, the naive tumors lacking HSV*tk* on the contralateral side showed no response to ganciclovir treatment, but displayed growth kinetics similar to those of controls. However, the mixed tumors on the ipsilateral showed retarded growth following ganciclovir treatment ($P < .05$), consistent with a local bystander effect mediated by gap junction. Taken in aggregate, the above results suggest the involvement of the immune system in the observed regression of tumor at distant sites.

DISCUSSION

The *in vitro* bystander effect is mediated, at least in part, by metabolic cooperation, which involves the passage of small cytotoxic molecules via gap junctions formed between physically apposed cells.[5–8,11] Cells that express HSV*tk* can phosphorylate ganciclovir, thereby trapping it within the cell. Autoradiography has shown that radioactive ganciclovir products can pass from HSV*tk*$^+$ cells to adjacent HSV*tk*$^-$ cells that are in physical contact, but not to HSV*tk*$^-$ cells that are physically isolated.[5] An alternative mechanism in which naive tumor cells phagocytose apoptotic vesicles produced by dying HSV*tk*$^+$ cells has been suggested.[4] These apoptotic vesicles may contain cytotoxic products, although the identity of the cytotoxic molecules in the vesicles is not clear.

In addition to events that are important *in vitro*, other factors may contribute to the bystander effect *in vivo*. As reported earlier, HSV*tk* transduction coupled with ganciclovir administration can cause damage to tumor vasculature, either as the result of direct endothelial cell transduction or as the consequence of cell killing by a local bystander effect.[12] Because angiogenesis is crucial to continued tumor growth, the damaged tumor vascular network can cause ischemia and inhibit tumor growth. There also is an increasing body of evidence implicating the immune system as a significant participant *in vivo*. First, a correlation has been described between regres-

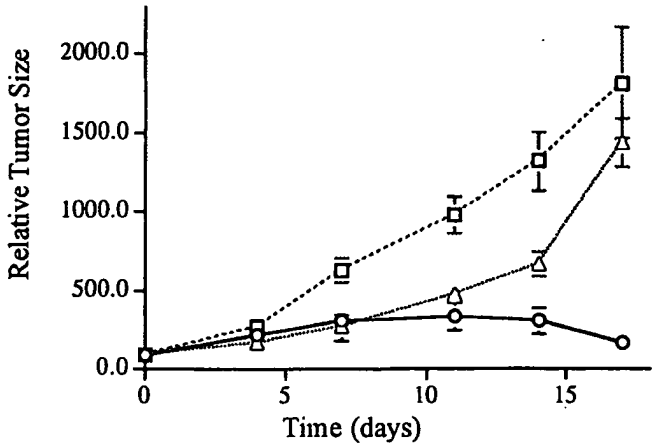

Figure 4. Suppression of the distant antitumor bystander effect in the contralateral flank by dexamethasone. Twelve nude mice were injected bilaterally with 5×10^6 UM SCC29 cells. Once tumor cells achieved a size of 0.5 to 1.0 cm, the tumors in the left flank were injected with 2×10^7 PA317 cells or NIH 3T3 cells. After 2 days, the mice were injected with ganciclovir alone or with ganciclovir plus dexamethasone (625 μg/kg body wt). The tumor size in the contralateral flank in each case was followed as a function of time. Injection of PA 317 cells followed by ganciclovir treatment alone (open circles); injection of PA 317 cells followed by treatment with ganciclovir plus dexamethasone (closed triangles); injection of NIH 3T3 cells followed by treatment with ganciclovir alone (open squares).

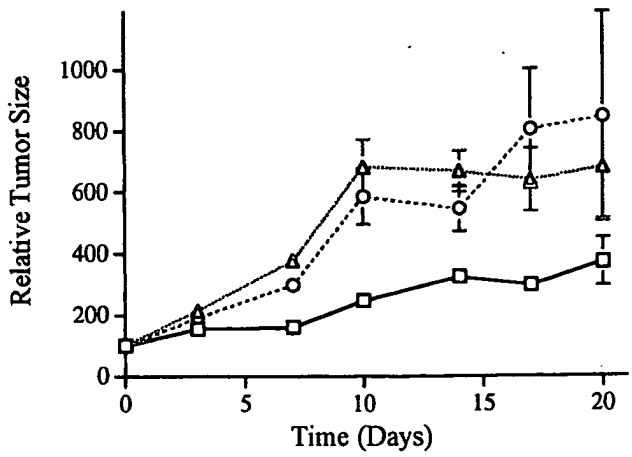

Figure 5. Absence of a distant bystander effect in SCID-Beige mice. Twelve SCID-Beige mice were injected subcutaneously in the left flank with 5×10^6 cells in a 1:3 ratio of UMSCC29*tk*:UMSCC29 cells. The same mice were injected with 5×10^6 naive UMSCC29 cells in the right flank. After the tumors reached an average size of 0.5 to 1.0 cm, the mice were injected intraperitoneally with ganciclovir as before. The tumor sizes in the ipsilateral flank (open squares) and contralateral flank (open triangles) were followed as a function of time. As a control (open circles), 12 mice were injected with naive tumor cells and treated with ganciclovir.

sion of metastatic lesions and the infiltration of macrophages, and $CD4^+$ and $CD8^+$ T-lymphocytes.[13] Second, the introduction of a foreign antigen, such as a hygromycin phosphotransferase/HSV*tk* fusion protein, can elicit an immune response and retard implanted tumor cell growth in a rat glioblastoma model.[14] Third, following HSV*tk* transduction into tumors and a subsequent ganciclovir administration, immunity can develop against the parental tumor type.[15] Fourth, treatment with HSV*tk*-modified cells can elicit a cytokine response.[16,17] Fifth, introduction of genes encoding both HSVTK and GM-CSF into tumor cells produced short term immunity against parental cells and an enhanced therapeutic response.[18]

The data in this report provide further supportive evidence for the involvement of the immune system in the *in vivo* bystander effect. In experiments in which naive tumors were grown in the flank opposite to that containing a tumor comprised of HSVtk^+ and HSVtk^- cells or of a naive tumor injected with producer cells or HSVtk^+ packaging cells, growth of naive tumors on the contralateral flank was impaired following ganciclovir administration. This is attributed largely to an immune response, since the naive tumor cells were physically separated from HSVtk^+ cells on the ipsilateral side. Although migration of HSVtk^+ cells to the contralateral side has not been ruled out formally, this possibility is unlikely because it would require the migration of a large number of cells to be effective. Similarly, neither metabolic cooperation nor involvement of apoptotic vesicles or damaged vasculature are likely mechanisms since all of their actions would be local. The most likely mechanism to produce an effect at a distance from the primary tumor is a systemic one that is mediated by an immune response. This proposition is supported by the observed enhanced lymphocyte infiltration in regressing tumors. The presence of increased numbers of lymphocytes on both the ipsilateral and contralateral sides suggests that tumor regression or retardation of growth involves immune participation on both flanks, although the relative impact on each flank might be different. Administration of dexamethasone, an immune suppressor, allowed the tumors on the contralateral side to grow more vigorously than without dexamethasone treatment. However, administration of dexamethasone did not affect retardation of tumor growth on the ipsilateral side. This difference might be due to a strong local bystander effect on the ipsilateral flank, largely independent of an immune response.

The data implicating an immune response in nude mice was unanticipated since nude mice are immunocompromised primarily due to a deficiency in mature T-lymphocytes. However, they do have functional NK cells as well as monocytes and macrophages. They also can produce antibody against non-protein antigens.[19] Also, older nude mice may develop some functional T-lymphocytes because of extrathymic T-cell maturation. Thus, in principle, nude mice have the capacity to mount an immune response, albeit an incomplete one, against implanted tumor cells. It is noteworthy that the nude mice used in this study were obtained from Harlan Sprague-Dawley. Although originally listed as Balb/c-*nu*, these mice were outbred in the past, and now less than

50% of loci examined are of Balb/c origin (Harlan Sprague-Dawley, personal communication). Thus, although these mice may be useful for growing human tumors, they also appear capable of mounting an antitumor response when appropriately challenged. No such response was evident in SCID-Beige mice. Interestingly, there was no long term immunity in the nude mouse model when mice whose ipsilateral and contralateral tumors had completely regressed were rechallenged with naive tumor cells. In all cases, the tumors grew unabated (data not shown).

The detailed mechanism of immune response remains unclear. We do not yet know the identity of the immune cells which mediated the response, nor do we know why ganciclovir treatment was essential for triggering the response. One possibility is that death of HSV*tk*-modified cells and their adjacent tumor cells attracted activated monocytes/macrophages and/or NK cells. Both of these cell types could potentially contribute to the distant bystander effect. Compared with immune competent mice, nude mice have an enlarged monocyte/macrophage pool.[20] Furthermore, systemic activation of macrophage has previously been shown to be a possible approach to suppress tumor metastasis.[21]

Although retroviral-mediated gene delivery into solid tumors is an attractive approach, it has several disadvantages. There is risk of producing replication competent virus by recombination, and there is potential risk of insertional mutagenesis. Transduction efficiency is low with only 0.1–1.0% of tumor cells transduced at the site of injection.[1] Lastly, immunity against retrovirions or producer cells will not allow repeated vector injection. An unanticipated finding was that PA317 packaging cells were highly effective in mediating the bystander effect *in vivo*. *In vitro*, UM SCC 29 cells were resistant to ganciclovir, whereas PA317 cells were not. When co-cultured, both cell populations were killed (Fig 1), indicative of metabolic cooperation. Intratumoral injection of HSV*tk*$^+$ cells that are nonproducer cells, such as PA317 cells, and that have the capacity to undergo metabolic cooperation with the tumor cells would eliminate the risk of producing replication-competent retrovirus and the risk of insertional mutagenesis. Although such cells cannot deliver HSV*tk* genes into the target tumor cells, this deficiency apparently can be overcome by metabolic cooperation, which can be effective in facilitating local tumor cell killing after ganciclovir administration.

The data suggest that, locally, the bystander effect mediated by metabolic cooperation, plays an important role in dictating the degree to which tumors respond following introduction of HSV*tk* and administration of ganciclovir. Furthermore, the data suggest that antitumor response to tumors at distant sites may be mediated by a cellular immune response. The data are consistent with the contention that presentation of a foreign antigen, such as HSVTK, in proximity to tumor cell killing, can be sufficient to induce a systemic antitumor response. Introduction of HSV*tk* without ganciclovir treatment is insufficient; and ganciclovir administration alone is insufficient. Intratumoral injection of murine HSV*tk* producer cells or HSV*tk*$^+$ packaging cells, followed by ganciclovir treatment mediated a distant bystander effect, whereas intratumoral injection of parental NIH 3T3 cells plus ganciclovir had no effect locally or at distant sites. In aggregate, the data point to the need for a foreign antigen in the context of tumor cell killing to elicit the distant bystander response.

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